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Review

Insulin and wound healing

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ABSTRACT

Skin is a dynamic and complex organ that relies on the interaction of different cell types, biomacromolecules and signaling molecules. Injury triggers a cascade of events designed to quickly restore skin integrity. Depending on the size and severity of the wound, extensive physiological and metabolic changes can occur, resulting in impaired wound healing and increased morbidity resulting in higher rates of death. While wound dressings provide a temporary barrier, they are inherently incapable of significantly restoring metabolic upsets, post-burn insulin resistance, and impaired wound healing in patients with extensive burns. Exogenous insulin application has therefore been investigated as a potential therapeutic intervention for nearly a century to improve wound recovery. This review will highlight the important achievements that demonstrate insulin's ability to stimulate cellular migration and burn wound recovery, as well as providing a perspective on future therapeutic applications and research directions.

Contents

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1. Burns and treatment

Burns are one of the most frequent skin injuries resulting from excess exposure to heat, caustic chemicals, solar, lumpectomy/mastectomy, or radiation [1]. Depending on the surface area and depth of a burn, healing times can vary significantly [2]. Most burns are treated using a dressing placed over the wound to isolate it from the environment. In more severe cases however, skin grafts, reconstructive surgery, or amputation may be required [3]. Rehabilitation following a burn can be difficult, painful, and time consuming. Burn wound treatments must therefore be initiated at the earliest possible opportunity to improve recovery and reduce the burden on healthcare resources.

Presently, burn treatments encompass a wide range of approaches depending on the condition of the burn. The majority of partial thickness burn wounds can be treated with a range of dressings including silver impregnated hydrocolloids to more traditional based materials such as white petrolatum coated gauze dressings [4]. These materials help prevent entry of foreign debris, maintain a moist wound healing environment and absorb excess exudate from the wound [5,6]. These materials are also widely available, affordable, convenient, and remain stable for extended periods, making them a gold standard in treatment [7]. Gauze dressings however, have no inherent capacity to promote wound healing beyond the natural rate of healing, and can destroy *de novo* tissue during dressing removal [8]. Therefore, new dressing technologies have been explored to overcome this limitation and to improve patient recovery.

In advanced trauma centers, more advanced materials and therapeutics can be applied to aid burn wound healing when significant reconstruction is required. Biologicals can be referred to as both the biopharmaceutical, or the dressing itself. Biological wound dressings for example, consist of human, porcine, or cadaver tissue which is pre-treated to produce an acellular scaffold, mimicking the native dermis and basement membrane. The use of biological dressings may however, give rise to rejection, transmit diseases, and are typically a temporary solution [9]. Application of growth factors in wound healing treatments has subsequently been tested as a method of promoting faster recovery by stimulating skin cells to migrate and proliferate more rapidly than naturally [10]. Historically, growth factors such as epidermal growth factor (EGF) and transforming growth factor beta (TGF-β) have been tested both in the laboratory and in the clinic with varying degrees of success [11,12]. Unfortunately, biological dressings and growth factors are difficult to store for extended periods, come at a high cost, and are usually complex to manipulate, thereby limiting their widespread clinical and commercial success.

Alternatively, insulin has been reported to promote wound healing, but in contrast to other growth factors, is lower in cost, available in a highly pure crystalline form, and is compatible with most common biomaterials used in wound dressings and drug delivery devices. Integration of insulin within a burn wound dressing would therefore be a potentially effective method of promoting wound healing, while reducing cost. Within the following review, the application of insulin both topically and systemically has been discussed in a historical context to highlight how insulin could be used in future wound dressings, and clinical approaches, while highlighting areas for further improving our understanding of insulin mediated healing.

2. Therapeutic biologicals in wound healing

Biologicals are a class of recombinant medicines that include monoclonal antibodies, nucleic acids, and small or large molecular weight proteins [13–15]. Approximately 150 recombinant biopharmaceuticals have been approved by the Food and Drug Administration (FDA) and the list of submissions and approvals continues to grow [16]. Recombinant peptides are an increasingly important therapeutic intervention against a variety of medical conditions including diabetic, oncologic, cardiovascular, immunosuppressive and gastroenterological diseases [17–21]. Growth factors are a subclass of biologicals that have the ability to stimulate, or inhibit cellular division, differentiation, migration, or gene expression in cells [22]. Growth factors can act in an autocrine, paracrine or endocrine fashion depending on the target receptor [23]. Once released, growth factors will either bind to their respective receptor, or become consumed by proteolytic enzymes resulting in degradation and inactivation. The action of growth factors is therefore strong and usually short-lived.

Biomedical researchers have examined the potential use of growth factors, especially in the field of wound healing [24]. Exhaustively investigated growth factors have included epidermal growth factor (EGF) [25], transforming growth factor beta (TGF-β) [26], and platelet derived growth factor (PDGF) [27]. Delivery of a growth factor to the wound allows the regenerative healing mechanisms to be initiated faster, as opposed to being released naturally by cells and tissues within the wound bed.

Unfortunately, the high cost of producing purified growth factors has prevented their integration into burn wound dressings. Beginning in the 1970s, growth factors were first harvested by processing tissue and/or blood samples from animals and humans [28]. Tissues would undergo mechanical and chemical treatments to yield small quantities of semi-pure growth factors. This approach was laborious and ineffective in sustaining large scale production. As molecular techniques advanced, recombinant technologies utilizing bacteria and yeast made it easier and safer to increase production [29]. As a result, the mean cost of growth factors such as EGF, TGF-β and other highly specialized growth factors can be upwards of $1500–10,000 USD per mg. Growth factor application in wound healing technologies has therefore been confined largely to experimental settings, and not commercial markets.

3. Insulin and wound healing

Insulin is a peptide hormone and growth factor with several physiological roles. It is primarily known to regulate blood glucose levels, but over the past century, insulin has played a quieter role in the field of wound healing. Since insulin can potentially help restore the integrity of damaged skin, it is of interest in the field of wound care, particularly given its low cost relative to other growth factors, and thus more likely to be considered for integration into wound dressings.

3.1. Discovery of insulin

The discovery of insulin was one of the greatest achievements of the 20th century. Prior to the early 1900s, medicine offered little relief to those diagnosed with juvenile diabetes, and little was known about the physiology and function of the pancreas. Juvenile diabetics resulted in poor health outcomes and treatment typically followed one of two approaches. The first included a starvation diet developed by Frederick Allen, which limited the patient's intake of carbohydrates and reduced the incidence of glycosuria [30]. The second and more morbid therapeutic option was to provide no medical intervention [31]. In both cases, young patients would often suffer and die from cachexia, or diabetic ketoacidosis resulting from impaired metabolic regulation [32].

Frederick Banting and Charles Best successfully isolated insulin in 1921 from the porcine pancreas [33]. Systemic administration of extracted insulin demonstrated successful lowering of blood glucose levels in dogs, and signified the introduction of a new therapy for juvenile diabetes.

Soon after the discovery of insulin, stability issues became apparent. Without access to fresh insulin, the peptide would degrade and fail in controlling serum glucose levels. In an attempt to correct this problem, John Abel in 1926 [34] co-crystallized insulin with zinc salts to form a stable hexamer. Schlichtkrull refined the formulation conditions, and identified a reliable and reproducible method of crystallizing insulin. This was an important step because a crystalline form enabled longer periods of storage without loss of bioactivity. Schlichtkrull also discovered that hexameric insulin favorably complexed with either two, or four zinc atoms stabilizing insulin for extended periods [32], allowing Sanfer to determine the primary amino acid sequence of the peptide nearly three decades later in 1955 [35], and Steiner to identify the biosynthetic pathway in 1967, making it subsequently possible to partially synthesize insulin. Steiner discovered that insulin is first synthesized as a proinsulin precursor, requiring post-translational modification to become fully active [36]. Two years later, Hodgkin and colleagues described the three-dimensional structure of insulin using X-ray crystallography, allowing for structure and function relationships to be completed [37]. These discoveries culminated with the introduction of recombinant technologies, permitting industrial-scale production, resulting in reduced cost and increased clinical availability [38]. Today, insulin is prepared in a number of highly stable and pure forms for nearly $1USD/mg.

3.2. Insulin biosynthesis and structure

Insulin is one of the most highly conserved peptides found in vertebrates and generally differs by 1–3 amino acids between species [39]. Naturally, insulin is biosynthesized by β-cells of the islets of Langerhans, clustered within the pancreas. The islets are supported by a highly vascularized and densely innervated network that provides accurate sensing, and control of blood glucose levels [40]. Normally, the pancreas is capable of producing up to 200 U (8 mg) insulin per day, of which only 50 U (2 mg) is usually secreted [41]. Within the islets, insulin is synthesized as a single-chain precursor, known as preproinsulin [42], comprised of several segments including: an N-terminal signal sequence of 24 amino acids, followed by the insulin B-chain, an Arg–Arg sequence, a connecting C-peptide, a Lys–Arg sequence and the insulin A-chain [40]. These signaling sequences are necessary to direct the secretion of the preproinsulin through the endoplasmic reticulum and Golgi bodies so that further post-translational modifications, such as the cleavage of the C-peptide, can take place [43,44]. Fig. 1 illustrates the structure of proinsulin produced in the pancreas.

Post-translationally modified insulin has molecular weight of approximately 5800 Da and consists of an A-chain with 21 and a B-chain with 30 amino acid residues, held together by 2 disulfide bonds. Fig. 1 shows the structure of proinsulin undergoing proteolytic cleavage of the C-chain. After modification, insulin complexes with zinc and forms crystalline granules within β-cells where it is sequestered until time of release [45]. Crystallization is a key facet in ensuring that insulin can be preserved for extended periods within the body, but released when needed.

Once released into systemic circulation, insulin is degraded by four different mechanisms, including degradation by the insulin-degrading enzymes (IDE), and by being bound and internalized by the insulin receptor (IR) [46–49]. These mechanisms ensure that prolonged insulin stimulation can be avoided and tight regulatory control enabled.

3.3. Insulin recombinant synthesis and crystallization

Industrially, insulin is produced using recombinant DNA technologies with genetically modified yeast and bacteria [50]. A recombinant approach alleviates the need for animal sourced organs, safeguarding against the transmission of animal-to-human pathogens, and is a cost-effective approach in large-scale production [51]. Purified insulin is crystallized at pH 5.8–6, by adding zinc salts [52]. Insulin also complexes and precipitates with cobalt and cadmium salts, however these preparations are not intended for clinical use [53]. The crystallization process is therefore a key step in stabilizing the peptide.

Crystallization of insulin also retards the dissolution rate under physiological conditions. For example, four-zinc hexameric insulin dissolves slower than non-complexed monomeric insulin, thereby allowing diabetic patients to maintain near constant serum levels of injected insulin for prolonged periods [54]. Regulation of blood sugar levels is then enabled through sleep periods by avoiding multiple injections. The rate limiting step in the dissolution mechanism is liberation of the zinc atom, which in turn releases insulin monomers that then bind with the insulin receptor (IR) [52].

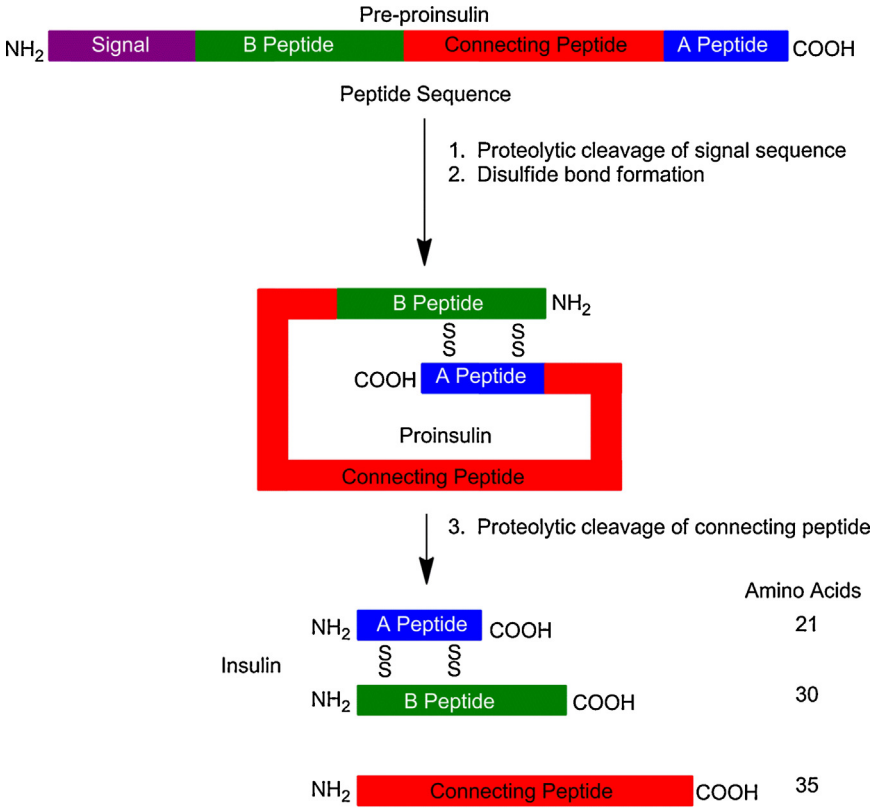

Fig. 1 – In the top image, preproinsulin is presented prior to folding, along with the signal sequence. After proteolytic cleavage of the signal sequence, the formation of disulfide bonds facilitates the folding of the peptide into the proinsulin state. After proteolytic cleavage of the connecting peptide, the A peptide (21 amino acids) and B peptide (30 amino acids) remain, held in place by the two disulfide bonds, forming insulin. The connecting peptide (35 amino acids) is left to be recycled in the cytosol.

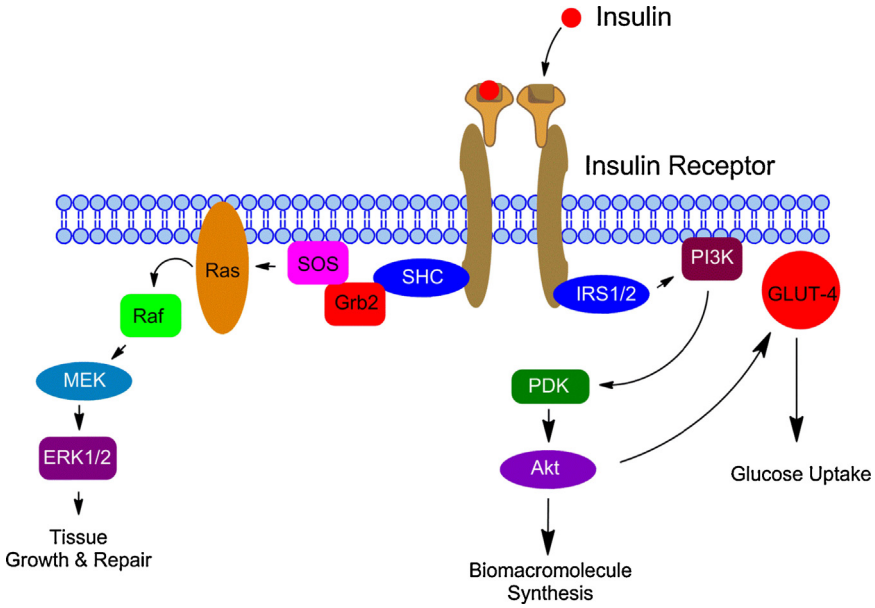

Fig. 2 – Insulin receptor and the major signal transduction pathways used in tissue repair, biomacromolecules synthesis and glucose uptake.

3.4. *Insulin receptor*

The insulin receptor (IR) is amongst the most ubiquitous cell surface receptors in the body. The IR belongs to a family of receptor tyrosine kinase (RTK) transmembrane signaling proteins, which include the insulin-like growth factor-1 (IGF-1), and EGF and PDGF receptors [55]. Insulin receptors are found on myocytes, erythrocytes, adipocytes, granulocytes, lymphocytes and in brain tissue at between 4 and 30×10^4 binding sites per cell [56–60]. IR binding sites have also been identified on both skin fibroblasts and keratinocytes in separate high and low affinity binding populations [61,62]. Fibroblasts have approximately 1.1×10^3 high affinity and 3×10^4 low affinity insulin binding sites per cell [63]. Keratinocytes have nearly 6×10^3 high affinity and 8×10^4 low affinity binding sites per cell. Specific to keratinocytes, the IR is responsible for inducing the expression of keratins 1 and 10 during calcium-induced differentiation and conversely inhibits the activation of the IGF-1 receptor which has an inhibitory effect [64]. The presence of high and low affinity binding sites allows the IR to respond variably in response to different insulin concentrations in the blood [65].

Structurally, the IR is initially synthesized as a proreceptor and cleaved to form a 350–400 kDa heterotetrameric receptor composed of two α-subunits (135 kDa each) and two β-subunits (95 kDa each) joined by disulphide bonds in a β–α–α–β configuration as illustrated in Fig. 2 [66]. The proreceptor is approximately 1340–1382 residues in length and can be spliced in more than one way [67,68]. The α-subunits are located near the N-terminal domain and constitute the extracellular [69] whereas the β-subunit contains a transmembrane domain, an intracellular region which contains the tyrosine phosphokinase, ATP-binding site and a tyrosine autophosphorylation site [70]. Other key regions of the IR include a leucine-rich repeat domain, a cysteine-rich region, a second leucine-rich repeat domain, and three fibronectin type III domains which help to maintain the conformational shape of the receptor [71].

During receptor activation, insulin binds to one of two binding sites on each of the αβ monomers called, site 1 and site 2 [71]. When insulin first binds to site 1, a high-affinity cross-linking reaction takes place, allowing 2 to become accessible for ligand binding to activate the receptor [72,73]. This binding regime means that IR demonstrates a negative co-operativity, meaning that as one ligand binds to the receptor, the IR affinity for a second ligand will decrease. Once bound, the α-subunit which normally prevents autophosphorylation of the β-subunit, is inhibited causing the kinase activity of the β-subunit to transphosphorylate, change conformation and increase its own kinase activity [74]. Interestingly, IR and IGF-1 receptors form functional hybrids which help elucidate why IGF-1 and insulin can act on each other's respective receptors [74].

After insulin binds to the IR and receptor dimerizes, a series of downstream signaling events follows. The signaling cascade begins with the phosphorylation of intracellular tyrosines located on the β-subunits of the receptor. The phosphorylated tyrosines act as docking sites, permitting the SHC protein found in the cytosol to bind *via* their Src-homology-2 domains [75–77]. The signal is then transduced *via* a series of messenger molecules including Grb2 (growth factor receptor-bound protein-2), which recruits SOS (son of sevenless), to activate Ras [78]. After activation, Ras carries the signal *via* Raf, MEK (members of the GTP-binding protein family) and ERK. ERK then translocates into the nucleus where the cell is given the command replicate DNA and proliferate [75]. This in turn, leads to tissue repair and wound healing.

IR activation also stimulates biomacromolecules synthesis and glucose uptake, two functions that are necessary to support tissue repair. Using the same phosphorylated tyrosines, insulin receptor substrates 1/2 (IRS1/2) dock to the receptor and transduce the signal *via* another set of downstream signaling messenger molecules including PI(3)-kinase, PDK and Akt. Phosphorylation of PDK and Akt surmounts in the synthesis of structural proteins, glycogen and the breakdown of fats. This helps with the secretion of biomacromolecules needed to repair damaged tissues, and provide enough energy to support cellular replication. Akt also activates a glucose transport molecule GLUT-4, which facilitates glucose uptake [76,79]. Phosphorylated Akt, can also phosphorylate eNOS and enhance NO (nitric oxide) production, leading to increased blood flow, cell survival, morphogenesis, and angiogenesis [78]. Other tyrosines found within the kinase domain begin to regulate the catalytic activity of the β-subunit as well [76]. Although the exact mechanism of insulin induced wound healing at the receptor level is yet to be fully elucidated, the insulin receptor does play a major role in tissue regeneration and the acquisition of biomacromolecules needed to support these activities. Moreover, the precise mechanism by which new blood vessels are formed during the application of insulin in severe burn wounds still remains unknown.

Soon after activation, the IR is quickly inactivated through two major pathways. The first includes internalization of the receptor which becomes sequestered from the plasma membrane and concentrated into heterogeneous, non-lysosomal tubulovesicular structures called endosomal apparatuses [80]. Once within the endosomal apparatus, the IR undergoes receptor sorting, recycling and degradation [80]. The second method includes protein tyrosine phosphatases that dephosphorylate the receptor and its substrates, resulting in receptor inactivation [81]. IR inactivation can be variable depending on the nature of the stimulus.

4. Early evidence of insulin affecting wound healing

Insulin's role in wound healing can be traced back to the early 20th century, to surgeons who observed differences in healing rates amongst diabetics and non-diabetics recovering from surgery [82]. In diabetics, wounds would fail to re-epithelialize normally, exposing open wounds to the environment, and resulting in infections. As the infection spread to the blood, death would soon follow. After the discovery of insulin in 1921, Thalhimer was the first to report the application of insulin to treat postoperative (nondiabetic) acidosis [83]. At the time, Thalhimer had been monitoring three patients who had undergone surgery and were suffering from severe vomiting and post-operative metabolic imbalance. Thalhimer reported that there was strong evidence linking acidosis with the development of severe infection and death. After injecting

three patients with glucose and 10 units of insulin, the symptoms of acidosis cleared, and the metabolic balance was restored. Improved recovery for the patients was evidence for the role of insulin regulating tissue repair. Foster in 1925 was also attempting to treat similar cases of diabetic acidosis by administering 30–75 units of insulin [84]. The mortality rate from infection in diabetics was reduced from 40 to 12% in those patients receiving insulin therapy intravenously amongst 20 patients. Thalimer and Foster observations provide early indications of insulin's ability to improve wound healing, and thus enhance postoperative health in diabetics.

Schazzilo and Ksendowsky used radiographic and histological techniques in 1928 to investigate the effects of insulin on bone fracture repair. Using a rodent animal model, 1 unit of crystalline insulin was injected into test animals, resulting in faster healing compared to blank control specimens of 5 animals each [85]. Soon after 1930, Joseph used insulin therapy to treat cardiac patients with non-diabetic bed sores. Joseph administered 10 units of insulin once daily to 5 patients, and observed improvements to the wound of 50 to nearly 100% after 14 days of therapy [86]. Rabinowitch later studied 250 consecutive surgical cases of gallbladder disease, which included a subset of 50 diabetic patients. Rates of death in both diabetic (4%) and non-diabetic (5.5%) patient pools showed that insulin reduced the risk of post-operative wound infection to a level comparable with normal patient recovery [87]. Stuck in 1932 examined the effect of exogenous administration of 1.5 units insulin intravenously to promote faster bone healing in rabbits with fractured left fibulas. Faster calcification of the callus in the presence of insulin was reported between 14 and 28 days *versus* the control group, and while bone morphology between the insulin and control groups did not differ, those treated with insulin healed faster [88].

4.1. *Insulin-wound healing research: 1940s–1980s*

Much of the research during the early part of this period was directed toward bone fracture healing before refocusing on soft tissue injuries. Lawrence et al. [89] studied the effects of insulin on nitrogen balance in hypophysectomized rats, postulating that insulin was responsible for nitrogen retention related to the uptake of amino acids, synthesis of proteins and inhibition of protein catabolism. These three factors are important to tissue repair following damage or injury. The study compared hypophysectomized rats treated with, and without insulin, along with non-hypophysectomized rats. Hypophysectomized rats receiving insulin responded positively to the insulin treatment and demonstrated a linear relationship in stimulating protein synthesis with dose. Lawrence concluded that insulin could therefore be a potent growth factor in the absence of pituitary signaling molecules, and potentially in tissue repair. Later, Stunkle and Wray examined the effect of long-acting insulin on the rate and quality of recovery from fractured tibias in non-diabetic and diabetic rats [90]. This was one of the first studies to utilize long-acting insulin, containing two arginine amino acids added to the C-terminal of the B chain and a substitution of asparagine 21 on the A chain with glycine [91]. The principle was based on lowering the isoelectric point to pH 4, so that upon injection, insulin would remain crystalline under normal physiological pH and dissolve more slowly with time. The authors concluded that there was no significant difference in tensile strength of repaired tibias between treated and control animals [90]. Unfortunately, the authors did not present findings related to the time to heal in control and treatment groups. In the same year, Gregory tested the effect of insulin on bone healing, by performing a simulated bone fracture on male albino rats [92]. A hole was drilled through the lateral aspect of the femur with a dental bur, cutting into the marrow. Following the injury, 40 units/kg of protamine-zinc insulin (intermediate-acting insulin) were injected every 24 h in 31 test animals and compared to 31 control subjects. The application of insulin showed no effect on bone strength, weight gain or histology [92]. Like the previous study, this study was not designed to examine the time to heal the defect in both control and insulin treated groups, only the break strength after insulin therapy. At this point, insulin therapy for bone regeneration in rabbit and rat models did not appear to improve bone strength after recovery and provided contradictory results to studies performed in the late 20s and 30s.

In the late 1960s, interest in using insulin for soft tissue wound healing was gaining popularity in both laboratory, veterinary and some human studies. In 1966, Paul described a case report on a 56-year-old diabetic woman who had developed gangrene which was spreading throughout the rest of her foot [93]. After amputation, the stump became infected with *Staphylococcus pyogenes*, releasing puss and eventually becoming necrotic. After repeated dosing with cloxacillin and erythromycin, no improvement in the infection was noted and the wound continued to heal poorly. Gauze soaked with 20 units of soluble insulin was then applied to the wound and covered. The dressing was changed twice daily and the injury healed within 3–4 day. Paul concluded that insulin may have helped to promote glucose utilization in tissues, resulting in improved healing and immediate clearing of the infection. Following this study, Rosenthal and Brooklyn examined the acceleration of primary wound healing by insulin in male albino Wistar rats [94]. A 4 cm vertical incision through the musculofascial layer, including the peritoneum was made on the left side of the upper part of the abdomen. Protamine zinc insulin suspensions were given daily for 2–3 days prior to the surgery, and then daily. Insulin treated animals showed a significant increase in wound tensile strength of nearly 15% over that of controls, 7 days after surgery, and weight gain of nearly 7-fold. The authors concluded that the insulin must have been stimulating protein synthesis increasing the wound tensile strength in animals receiving insulin treatment. Belfield et al. (1970) developed a cream (Ulcerin) containing 10 units of insulin, that was applied to surgical wounds, fresh bites and lacerations on cats and dogs [95]. In non-infected wounds, no noticeable wound healing response was observed, however animals with abscesses, ulcers, or infected surgical wounds had a strong response to ulcerin in as little as 24 h in some cases. After nearly 100 animal cases, the cream was moved into the clinic for testing. Ulcerin strength was adjusted from 10 to 80 units of insulin and was applied topically twice daily. Healing of ulcerated wounds began to appear within 48 h along with new tissue growth in the treatment of 20 animals. The authors suggested that clinically, Ulcerin was helping to normalize cell

permeability, increase vascularization, reduce exudation, arrest bacterial growth, enhance phagocytosis, stimulate proliferation, decrease local tissue hypoxia, eliminate edema and increase wound contracture.

Udupa and Chansouria (1971) examined the biochemical and histological aspects of insulin-mediated wound healing [96]. Rats were dosed daily with 0.02 U/g of body weight, administered subcutaneously. The experiments included 25 insulin treated rats and 25 controls, and continued for approximately 3 weeks in which insulin therapy ceased 5 days before sacrificing. Linear musculoperitoneal wounds measuring 5 cm in length were made and sutured closed. Protamine zinc insulin significantly increased the bursting strength of experimental abdominal wounds in rats, along with an earlier appearance of collagen fibers which were dense and better oriented compared to control animals. Goodson and Hunt examined wire mesh cylinders implanted subcutaneously into diabetic rats [97]. Rats were given insulin, either immediately or in intervals after the implantation procedure. Wound healing was assessed by measuring the concentration of hydroxyproline in the tissue which had grown into the cylinders after 21 days. Insulin therapy provided during inflammatory and proliferative phases resulted in improved recovery as compared to delivery later in the healing process. Weringer et al. (1982) continued to study the effect of insulin on wound healing in diabetic mice [98]. Diabetic and non-treated C57B1/6 mice received small dermal wounds in the ear up to 40 h after injection of insulin, or placebo. The wound tissue was excised 8 h later and the presence of capillaries, fibroblasts, and collagen, studied microscopically. Diabetic mice showed impaired healing, whereas diabetic mice provided with insulin responded similarly to non-diabetic controls by reducing the mean level of hyperglycemia experienced after wounding. No detectable difference in the duration of healing was observed between insulin-treated and non-insulin treated diabetic mice. The authors concluded that the mild reduction in hyperglycemia does support the hypothesis that insulin is necessary in an adequate wound healing response.

4.2. *Animal and human wound healing studies – 1980s to present*

Human and animal wound healing studies in the last 30 years have been focused on understanding and controlling the intense biochemical and physiological changes that follow post-burn. The majority of these studies have been directed toward potential clinical approaches and outcomes, as opposed to identifying the precise biochemical mechanisms. From these reports, it is clear that hyperglycemia and insulin resistance are widely observed in severely burned patients, reducing their chances of a successful recovery. Providing insulin delivery as part of a therapeutic strategy on the other hand, has shown to improve wound recovery, although the precise mechanisms remain to be fully elucidated in future studies.

4.2.1. *Clinical studies*

Wolfe and colleagues (1995) tested the effectiveness of long-term insulin infusion to alter protein kinetics in skeletal muscle in severely injured burn patients [99]. The hypothesis tested was that protein anabolism could be altered by an increased rate of transmembrane amino acid transport upon stimulation by insulin infusion. Nine patients with severe burns were given 7 days of high-dose insulin in a random small trial. Exogenous insulin delivery was found to stimulate protein synthesis by 50% in the wound, as compared to controls, however varied greatly between patients. Three years later, Wolfe's group conducted a similar study whereby six patients with burns to greater than 40% of total body surface area received insulin plus glucose infusions for 7 days [100]. The aim was to show that limb protein anabolism and improved wound healing could be achieved. Patients were given 25–49 U/h along with dextrose to maintain euglycemia, and burn wound donor-sites were biopsied and analyzed. Insulin, along with glucose was shown to reduce healing times from 6.5 ± 1.0 days to 4.7 ± 1.2 days during insulin infusion. Laminin staining of the biopsied tissue revealed intense staining along the basal lamina and blood vessels, together with an increase in collagen type IV after combined insulin therapy. The study proved to be clinically significant because it highlighted that a combined insulin–glucose therapy can be used to greatly improve the quality of wound matrix formation, and reduce the healing time by nearly 30%.

Wolfe and colleagues in 1999 continued to examine wound healing in animal models to determine if insulin or growth hormone could stimulate protein anabolism in male New Zealand white rabbits [101]. Four groups including a control ($n = 10$), a low-dose insulin ($n = 5$), a high-dose insulin ($n = 5$) and a growth hormone group ($n = 5$) were subjected to a partial thickness, scald burn to the ear. A L-[ring-$^{13}C_{16}$]phenylalanine trace was infused to measure muscle protein synthesis, while insulin was infused at either 0.6, or 2.3–3.4 mU/kg/min. Low or high dose insulin inhibited protein proteolysis, while growth hormone had no net effect. Using the same experimental protocol, co-administration of amino acids, including radio-labeled phenylalanine and proline, and insulin was tested to determine if the net protein balance in skin wounds could be improved. Rabbits were injured with a partial thickness scald to the ear and treated with 2.5 mU/kg/min insulin and a combination of lysine, valine, phenylalanine, histidine, threonine, methionine, tryptophan, alanine, arginine, glycine, proline, serine and tyrosine at doses ranging from 40 mg to 2.07 g [102]. The combination of both insulin and amino acid therapy caused a shift from protein catabolism, to anabolism in the skin wound from -6.5 ± 4.5 to 1.4 ± 5.2 μmol/100 g/h, compared to insulin, amino acids alone, or without any treatment at all. The authors concluded that there is an interactive effect of insulin and sufficient amino acid supply on protein metabolism in skin wound and that it is better when co-administered, rather than individually infused into animals. In a similar study, Gore et al. compared glucose or insulin administration on its effect on protein catabolism in 6 burned patients with burns covering up to 60% of their total body surface area [103]. Using a 2H5 phenylalanine and 15N alanine tracers, measurements were recorded 9 h after fasting, during an I.V. glucose infusion (30 μM/kg/min) and during a hyperinsulinemic (500 mIU/kg/min) period. Gore reported that when compared to fasting values, administration of glucose had caused significantly greater muscle

catabolism to supply recovering burn tissue with essential amino acids. Insulin in comparison, had decreased muscle catabolism and may be helpful in controlling metabolic fluctuations in severely burned patients. Systemic insulin administration is therefore a useful treatment in controlling muscle wasting and regulating metabolic activities during the recovery process.

In 2008, Wolfe's group [104] examined 30 pediatric patients with over 40% TBSA burns. They observed that hyperinsulinemia did not stimulate muscle insulin signaling and insulin resistance was accompanied by decreased insulin signaling and increased protein kinase C–B activation.

Van den Berghe et al. [105] conducted a study on 1548 patients admitted to an intensive care unit to determine if normalization of blood glucose with insulin therapy improves the prognosis. Patients were randomized and either received intensive insulin therapy or conventional treatment. After 12 months, it was observed that intensive insulin therapy reduced mortality during intensive care from 8 to 4.6%. Other important benefits were reported as well, with the authors showing that insulin therapy reduced both morbidity and mortality, irrespective of the nature of the illness or injury.

Jeschke and colleagues examined mechanisms for the role of insulin in promoting burn wound healing. It was reported that after severe burns, metabolic upsets associated with insulin resistance and impaired signaling can be addressed with insulin dosing leading to reduction in synthesis of proinflammatory cytokines and signaling transcription factors, combined with improved hepatic structure and function [106]. In contrast, hyperglycemia and insulin resistance can lead to increased morbidity and mortality [107]. The effect of intensive insulin therapy on mortality of 239 severely burned pediatric patients over 30% of total body surface area (TBSA) was examined [107]. Insulin therapy compared to the controls showed improvements in patient outcomes by reducing infections and sepsis, organ function, alleviation of insulin resistance and catabolic responses, and dampened inflammatory and acute-phase responses. Mortality was 4% for patients receiving intensive insulin therapy compared to 11% for the control group.

Hyperglycemia and insulin resistance following severe burns, were proposed to be associated with ER stress and unfolded protein response activation, leading to impaired insulin receptor signaling [108]. In a study comparing 27 burn pediatric patients to 36 healthy children, conducted for 466 days postburn, injury was shown to be associated with altered signaling pathways affecting insulin resistance, ER and sarcoplasmic reticulum stress, inflammation, and cell growth and apoptosis over the recovery period, confirming the results of earlier studies.

Kabalak et al. (2013) studied 23 severe burn patients with normal glucose levels, half of which received insulin [109]. It was observed that insulin treatment decreased pro-inflammatory proteins and serum triglyceride levels, incidence of sepsis and length of hospitalization, albumin substitutions and patient morbidity and mortality relative to the non-insulin treated patients. Increased hepatic proteins were observed in the same group.

Rezvani et al. (2009) conducted a randomized, double-blind, placebo-controlled trial to determine the effects of topical insulin on wound healing in 45 patients with non-infected acute and chronic extremity wounds [110]. Topical application of a 0.1 mL/cm^2 of 0.9% saline spray, or 10 U of crystalline insulin twice daily was used. The endpoint was complete wound closure. There were no significant differences in wound healing rates between control and treatment groups which could have been related to the experimental protocol where the solution was permitted to dry on the surface of the wound. This would have prevented insulin from penetrating and diffusing into the wound, resulting in no improvement to wound healing. Wilson et al. (2008) reported on the topical use of insulin to promote healing of problematic surgical wounds [111]. An 80 year-old female patient with a chronic non-healing wound following a laparotomy procedure, failed to heal, even after 3 weeks of negative-pressure vacuum pump dressing application. The patient underwent insulin therapy as a last resort method. The dressings were replaced with daily irrigation with 20 mL saline containing 2 U of soluble insulin for 7 days. At the end of the therapy, visible improvement was observed, along with no metabolic side effects. Fram et al. (2010) conducted a randomized trial on 20 pediatric burn cases with burns to more than 40% of total body surface area [112]. Blood glucose levels of one group were maintained between 80 and 110 mg/dL, and approximately 215 mg/dL for a second group, with intravenous infusions for 10–14 days. The findings concluded that intensive insulin therapy in pediatric patients can be performed safely with the ability to improve metabolic balance and to prevent insulin resistance after acute trauma. Scimeca et al. (2010) combined insulin chemotherapy with negative pressure wound therapy (NPWT) in treating complex wounds [113]. NPWT is used to assist in the development of granulation tissue by improving wound contracture, moisture and re-epithelialization. A 71 year old male diabetic patient who had received an emergency amputation, received NPWT in conjunction with an infusion drip of insulin at a rate of 40 mL/h following a dose similarly used by Wilson. Within 48 h, the wound base was 90% granular with neo-epithelialization visibly present. Tuvdendorj et al. (2011) also explored the use of intensive insulin-therapy in enhancing the healing of skin graft donor sites in burn patients by increasing the fractional synthesis rate of wound protein [114]. The randomized trial followed 13 control and 10 insulin treated pediatric patients with burns to greater than 38% body surface area, requiring skin grafting. Insulin was provided at a rate of 0.1 U/kg/h and the blood glucose level maintained at 80–110 mg/dL. The metabolic response 2–4 (early period) and 5–6 (late period) days postoperatively was examined. In the early period, intensive insulin therapy increased the fractional synthesis rate of wound proteins as determined by radiotracers administered during the insulin therapy to measure protein turnover rates. The significance of this study reveals that in skin graft donor sites, insulin therapy administered as early as possible, is able to increase fractional synthesis of proteins more effectively.

4.2.2. *Animal studies*

Wolfe's group in 2007 [115,116] examined the effects of localized insulin delivery *versus* intravenous administration as reported in previous studies. Local insulin–zinc was injected into the backs of dermatomed New Zealand white

rabbits, simulating a partial thickness wound to the skin [115]. The purpose was to determine if localized insulin delivery could accelerate wound healing as effectively as intravenous infusion, but without the effects of hypoglycemia and hypokalemia. Injections of 0.25 units insulin were directly placed into 5 sites around the wound, approximately 1–1.5 cm from the wound margin every other day. The treatment resulted in wounds healing in 11.2 ± 2.3 days as compared to controls that healed on average 15.1 ± 4.1 days [116]. Using a similar experimental protocol, they determined that DNA synthesis also increased by nearly 50% in wound tissue as compared to control animals [115]. Wolfe's research showed that although infusion of insulin is one method of improving wound healing in animal models, localized insulin delivery can be just as effective in accelerating wound closure. In 2011, the group revisited the insulin infusion model to determine if DNA and protein synthesis would increase with the presence of insulin [117]. Protein and DNA synthesis rates were up 9.0 ± 1.2 and $5.4 \pm 0.5\%$ per day, *versus* the controls which had rates of 6.4 ± 0.5 and $4.0 \pm 0.6\%$ per day respectively. As a result, they were able to demonstrate that intravenous and topical insulin delivery is capable of reducing wound healing times, and increasing both protein and DNA synthesis by wound tissue.

Wound repair of corneal tissues was also examined in diabetic rats to study insulin's role in improving re-epithelialization. Zagon et al. (2006) investigated whether intensive treatment with insulin could be used to maintain blood glucose levels at near normal levels in diabetic rats to facilitate delayed corneal wound healing [118]. Corneal abrasions were inflicted and followed with insulin injections to maintain blood glucose near normal levels after 9 and 11 weeks post-induction of diabetes. Healing and DNA synthesis in diabetic rats treated with insulin exhibited similar healing as control animals, suggesting that intensive insulin therapy prevents delayed wound healing on the ocular surface of diabetic rats. Zagon in 2007, reported similar results whereby insulin was topically applied to injured corneal surfaces [119]. Eye drops containing 1, 2 and 5 U of insulin applied 4 times daily for 7 days resulted in diabetic rats healing at a similar rate to that of non-diabetic test animals.

Zhang (2011) studied intensive insulin therapy in burn-induced acute lung injury in rats. Pulmonary microvascular endothelial cell dysfunction, decreased cell apoptosis and inhibition of acute lung injury was demonstrated at 12 h after the injury [120]. It was proposed that such therapeutic approaches can mitigate organ failure after burn.

Wang et al. (2012) examined the effects of full thickness burns to 30% TBSA in rats, on intestinal barrier damage [121]. It was proposed that glucose–insulin–potassium (GIK) therapy could protect the intestinal barrier from damage. Wang showed that the degree of intestinal damage in the insulin treatment groups was significantly lower than that of the control group. All indices including body weight showed improvement, which was proposed to relate to control of hyperglycaemia and regulation of intestinal expression of pro- and anti-inflammatory cytokine genes, providing a protective effect on intestinal tissue.

Jeschke (2011) reported that severe burns lead to hepatic apoptosis, endoplasmic reticulum (ER) stress, and activation of the JNK pathway causing metabolic dysfunction [122]. It was hypothesized that insulin attenuates these affects. A study was conducted with rats receiving burns to 60% TBSA. Following 24–48 h, insulin treated animals showed reduced hepatic ER stress, reversed structure/function changes in hepatocyte mitochondria, and attenuated expression of IL-6, MCP-1 and CINC-1 leading to reduced inflammation, compared to controls.

Chen and Zhang (2012) examined the application of topical insulin and inflammatory response following application of full-thickness excision wounds to mice [123]. Wound closure was reduced from 7 to 5 days in insulin treated animals. This was a result of suppressed neutrophil infiltration and decreased levels of MIP-2 expression within 48 h post-burn.

Zhang et al. (2013) examined burn-induced insulin resistance in mice [124]. Of interest was stress kinase activity and adipocytokine expression in brown adipose tissue. Down-regulation of mRNA expression of insulin signaling cascade proteins was observed, leading to the conclusion that mRNA levels of adipocytokine proteins that regulate lipid and glucose metabolism and energy expenditure were substantially affected by burn.

4.3. *Cell based wound healing studies – 1990s to present*

While human and animal trials were being conducted by the Wolfe group, insulin therapy was also being explored in animal and tissue-based studies to further examine the molecular events behind the role of insulin in enhancing the healing process. Yano et al. (1996) evaluated the effect of insulin on fracture and wound repair of bone at both the tissue and cellular level using parietal bones removed from 20 day old Sprague-Dawley rat fetuses and cultured *in vitro* [125]. After fracturing, specimens were placed in either medium with 10^{-6} mol/L insulin, or medium without insulin, and studied 12 days. Bones receiving insulin treatment showed complete bone gap restoration after the 12th day, along with a thick osteoid layer and several layers of osteoblasts. No osteoclasts were detected. In the control group, 6 bones did not bridge the gap, while the remaining 5 indicated some marginal bone growth. The frequency of bone bridging (gap restoration) was statistically significant in the insulin treated group, compared to the insulin-free controls. Yano concluded that insulin had promoted bone repair through the stimulation of bone-forming cells and inhibition of bone-resorbing processes. Huang et al. (1999) studied the effect of extracellular calcium, in combination with zinc, insulin and insulin-like growth factor 1 (IGF-1) on DNA synthesis in 3T3 fibroblasts cultured in serum containing 1.8 mM Ca^{+2}, stimulated with 0.75–2.0 mM Ca^{+2}, along with the addition of zinc (15–60 mM), insulin and IGF-1 [126]. Insulin, along with zinc had prolonged Ca^{+2} induced mitogen-activated protein (MAP) kinase activity and p70 S6 kinases, thereby promoting improved fibroblast growth.

Shanley et al. (2004) investigated the effects of insulin and leptin on *in vitro* wound healing of transformed human corneal epithelial cell monoloayer [127]. The aim was to identify cellular and intracellular signaling pathways involved in either proliferation, or migration events. Using a monolayer of human corneal epithelial cells (HCEC), a single scratch was made into the monolayer of cells. The scratched monolayers

were exposed to insulin and leptin and monitored every hour for 8 h. Western blot analysis of phosphoinositide 3-kinase (PI3-kinase) and MAP-kinase activation was conducted. Inhibitors such as Ly 294002 and wortmannin were used to prevent PI3-kinase and ERK 1/2 insulin induced activation as well. Shanley concluded that insulin alone facilitated wound closure of HCEC cell monolayers through the phosphorylation of ERK 1/2 and Akt and that their inhibition severely reduced insulin-induced wound healing. Insulin facilitated cell migration instead of proliferation through the lack of BrDU uptake as the scratch area became filled over the 8 h period. The importance of this study is that the authors showed that for small wounds, cell migration is the key driving force in wound healing whereas in larger wounds, proliferation plays a larger role. Musselmann et al. (2005) observed the effect of keratocytes (mature corneal stroma cells) co-cultured with three different growth factors including fibroblast growth factor-2 (FGF-2), insulin and platelet derived growth factor-BB (PDGF) on keratocyte proliferation and the maintenance of the keratocyte markers over 7-days at low and high seeding densities in serum-free conditions [128]. Proliferation was measured using [^{3}H]thymidine incorporation and by DNA content of the cultures. The expression of cytosolic aldehyde dehydrogenase and keratocan determined by Western blot, measured the degree of keratocyte phenotype expression during the course of the experiment. All growth factors stimulated proliferation and increased the DNA content of the cultures over the 6-day period. Insulin however, stimulated thymidine incorporation in a more consistent fashion, and attained the greatest accumulation of DNA across all cultures. Likewise, insulin maintained keratocyte morphology at the end of the study period more effectively than the other growth factors. The relevance of this study indicates that insulin may be an important element in maintaining the phenotype of proliferating cells in culture.

The cellular and molecular mechanisms of wound healing and vascular repair within the skin were examined by the Martins-Green group. Liu et al. (2008) studied insulin's angiogenic effects using a combination of *in vitro* and *in vivo* techniques [129]. C57BL/6 J mice were injected with 0.03 units of insulin per 20 μL saline in treatment groups and saline only in control groups every 24 h for 5 days. On day 6, skin samples were removed and histologically analyzed. Insulin injections stimulated the formation of microvessels, with significantly more branching as compared to control animals receiving saline injections. Cellular migration and DNA synthesis was measured using human microvascular endothelial cells (HMEC) treated with insulin concentrations ranging from 10^{-5} to 10^{-8} M. As well, insulin stimulated endothelial cell migration and tube formation independently of the VEGF/VEGFR receptor pathway. The molecular mechanism of action was found to be driven by the PI3-K, Akt, sterol regulatory element binding protein 1 (SREBP-1) and Rac 1 signaling proteins. Inhibition of any of these pathways resulted in the elimination of endothelial cell migration, tube formation and the development of microvessels. Liu et al. (2009) explored the cell and molecular mechanisms of keratinocyte function in similar *in vivo* and *in vitro* studies [17]. Using C57BL/6J mice, 7 mm excision wounds were made and treated with a topical application of 0.03 units of insulin. In addition, human keratinocytes co-incubated with insulin, were assayed using a cell scratch assay. Both studies demonstrated that topical insulin application to excision wounds and in human cell culture would result in the migration and differentiation of keratinocytes. Wounds in mice showed accelerated wound re-epithelialization more rapidly than controls. The tissue response in culture depended on the activation of the PI3-K and Akt pathway, followed by the activation of Rac1 and the integrin α3 and the ECM molecule laminin 332. The authors reported that this study helps to identify the critical pathways involved in skin wound healing and the importance that insulin could have in future biopharmaceutical wound healing therapies.

Topical insulin delivery has therefore been shown to be beneficial in stimulating re-epithelialization, angiogenesis and in the recovery of both acute and chronic wounds *in vitro* and *in vivo*. Wound healing technologies that could provide sustained and controlled delivery of bioactive insulin would be beneficial.

4.4. *Topical insulin delivery*

Historically, insulin application on wounds has been limited to only a few approaches. These approaches have included soaking bandages in insulin solutions, applying insulin containing ointments, and in some cases, subcutaneous injections. In all cases, these methods require frequent reapplication, and cannot provide sustained controlled delivery of bioactive insulin for extended periods. Hrynyk et al. (2010, 2012) reported that highly stable crystalline insulin could be effectively encapsulated within PLGA microspheres to provide sustained, controlled delivery for more than 3 weeks [130]. Bioactivity was assessed using a human keratinocyte scratch assay (HaCat), showing that keratinocyte migration was significantly faster than in the insulin-free control. Extended and sustained delivery of bioactive insulin from a stable crystal-based depot device was achieved, showing promise as a delivery mechanism for wound dressings. Later, crystalline insulin-loaded PLGA microparticles were combined into an alginate-PEG sponge wound dressing (ASD) [131]. The ASD successfully released insulin for up to 21 days and preserved bioactivity for at least 10 days, as demonstrated by a HaCaT scratch assay. The results showed that PEG-ASD can function as an effective long-term delivery platform for bioactive insulin release.

5. Conclusion

Wound healing is a complex series of events aimed at repairing damaged tissues and restoring the skin's integrity. Insulin, a potent stimulator of wound healing, has been reported for nearly a century to induce rapid recovery from severe wounds, as shown by a variety of human, animal and cell-based studies. Together with modern wound dressings, insulin can help in restoring metabolic upsets, and impaired cellular signaling in burn patients. In conjunction with stem cells, concurrent insulin therapy may focus differentiation toward specific cell types, or perpetuate self-renewal more effectively, however future research will be required to further

resolve the specific mechanisms involved. Insulin dosing is an effective and safe method of promoting faster wound recovery, at a significantly lower cost than alternative therapeutic growth factors.

Conflict of interest

Authors report no conflict of interest.

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